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How many species in the *Rhizopogon roseolus* group?

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Abstract —Species concepts in the *Rhizopogon roseolus* species group (*Boletales, Basidiomycotina*) are analyzed using nrDNA ITS sequence data. This group includes taxa traditionally placed in sect. *Rhizopogon* in subsect. *Rhizopogon* (stirps *Rubescens*) and subsect. *Angustipori* (stirps *Vulgaris*) along with many other European species. ITS sequence analyses separate the collections into numerous clades and imply the existence of five phylogenetic species.

Key words — internal transcribed spacer, nrDNA, taxonomy

Introduction

The genus *Rhizopogon* belongs to the order *Boletales* and comprises ca 100 species. Martín (1996) reported 21 species of *Rhizopogon* for Europe with *R. roseolus* (Corda) Th. Fr. sensu M.P. Martín cited as the most abundant. However, the taxonomy of *R. roseolus* has long been controversial (Smith & Zeller 1966, Groβ & Schmitt 1974) and current authors (Martín 1996, Grubisha et al. 2002) also disagree over its species concept.

The holotype of *R. roseolus* — an illustration by Corda (1831) of *Splanchnomyces roseolus* — shows basidiomes with a uniformly vinaceous peridium and white gleba. In the genus *Rhizopogon*, the peridium and gleba colour serve with chemical tests as the main characters used to describe new species. The absence of yellow in the Corda illustration caused many authors to describe new taxa. However, recent morphological analyses by Martín (1996) proposed that 36 taxa described by many different authors (Corda 1854; Boudier 1885; Karsten 1886, 1889; Fries 1909; Velenovský 1931, 1939; Vacek 1948; Soehner 1956; Svrček 1958; Smith & Zeller 1966; Pacioni 1984a,b) are synonymous with *R. roseolus.*

Smith & Zeller (1966) included some of these taxa in *Rhizopogon* subgen. *Rhizopogon* sect. *Rhizopogon* but in two different subsections and stirps

according mainly to spore size: a) subsect. *Rhizopogon*, stirps *Rubescens* with basidiospores ≥ 3 μm diam — e.g. *R. abietis* A.H. Sm., *R. luteorubescens* A.H. Sm., *R. pseudoroseolus* A.H. Sm., *R. roseolus* sensu A.H. Sm., *R. ventricisporus* A.H. Sm., and four *R. rubescens* varieties); and b) subsect. *Angustipori*, stirps *Vulgaris* with spores ≤ 3 μm diam — e.g. *R. vulgaris* (Vittad.) M. Lange.

Other authors, such as Groβ & Schmitt (1974), separated taxa according to spore volume ($Vm = 0.5 \times w^2 \times L$ (w = spore weight, L = spore length). Thus, Groβ et al. (1980) placed the following in the *R. roseolus* complex based on spore volume: A—*R. vulgaris* (V≈ 15 μm^3); B—"*R. vulgaris* var. *intermedius*" Svrček and *R. pumilionus* (Ade) Bataille (V≈ 30 μm^3); C— *R. luteorubescens* (V≈ 45 μm^3); D—*R. rubescens* (Tul. & C. Tul.) Tul. & C. Tul. var. *rubescens* sensu A.H. Sm. as synonymous of *R. roseolus*, (V≈ 60 μm^3); and E—*R. hymenogastrosporus* Soehner (with two types of basidiospores, the larger with a V≈ 240–280 μm^3).

In earlier preliminary molecular analyses (nrDNA ITS2 sequence cladistic analyses; cluster analysis of ITS-RFLP patterns produced using 5 endonucleases), Johanesson & Martín (1999) and Martín et al. (2000) found that *R. roseolus* DNA isolates show a high degree of variability even when obtained from basidiomes comprising the same collection. Furthermore, the different patterns obtained did not correlate to the different taxa proposed by Smith & Zeller (1966) or Groβ et al. (1980).

Based on morphological and ITS sequences, Grubisha et al. (2002) presented a *Rhizopogon* subgeneric taxonomic revision. In agreement with Johanesson & Martín (1999), they concluded that Smith & Zeller (1966) sect. *Rhizopogon* is not monophyletic and proposed 5 subgenera to accommodate Smith & Zeller (1966) sections, subsections, and stirps. However, Grubisha et al (2002) included only 10 taxa of Smith & Zeller's sect. *Rhizopogon.* The phylogenetic tree placed one *R. roseolus* (subsect. *Rhizopogon*, stirps *Rubescens*) collection, two *R. vulgaris* (subsect. *Angustipori*, stirps *Vulgaris*) collections, and one *R. burlinghamii* A.H. Sm. (subsect. *Angustipori*, stirps *Ochraceorubens*) collection together in one clade; Grubisha et al (2002) described this clade as subgen. *Roseoli.*

In recent years, ITS sequence-based analyses have proved very useful for re-addressing species concepts in *Rhizopogon* (Kretzer et al. 2003) and in revealing cryptic species in other *Boletales* taxa, such as *Pisolithus* (Martin et al. 2002) and *Coniophora puteana* (Hauserud et al. 2006).

Thus, the main objectives of our paper using phylogenetic analyses of ITS DNA sequences are:

1. To explore the molecular variability of *R. roseolus* sensu M.P. Martín by comparing taxa regarded as synonymous in Martín (1996) and correlating the variability with morphological, ecological, or geographical differences.

2. To clarify how closely related are the taxa referred by Smith & Zeller (1966) to *Rhizopogon,* sect. *Rhizopogon,* subsects. *Rhizopogon* (stirps *Rubescens*) and *Angustipori* (stirps *Vulgaris*).

Materials and methods

Material

This study is based on 1458 collections previously identified as *R. roseolus* (Martín 1996) and new collections found in the MA herbarium (RJB). Appendix 1, lists collections selected for sequencing. Many collections were previously proposed as synonymous to *R. roseolus* in Martín (1996): *R. duriusculus* Velen., *R. gigasporus* Pacioni, *R. graveolens* f. *pomaceus* Vacek, *R. inodorus* Velen., *R. lapponicus* P. Karst., *R. minor* Velen., *R. luteorubescens*, *R. mohelnensis* Velen., *R. pseudoroseolus*, *R. pumilionus*, *R. roseolus* f. *amygdaloporus* Th. Fr., *R. roseolus* f. *aberrans* Th. Fr., *R. roseolus* var. *foetens* Svrček, *R. rubescens*, *R. rubescens* var. *ochraceus* A.H. Sm., *R. rubescens* var. *pallidimaculatus* A.H. Sm., *R. sardous* Pacioni, *R. tenuisporus* Velen., "*R. tenuisporus* var. *intermedius*", *R. ventricisporus,* and *R. vulgaris*.

Other representatives of sect. *Rhizopogon* (*R. abietis*, *R. luteolus* Fr., *R. ochraceorubens* A.H. Sm., *R. ochroleucoides* A.H. Sm.) were included, with taxa from other *Rhizopogon* sections selected as outgroups — sect. *Amylopogon* (*R. atroviolaceus* A.H. Sm., *R. ellenae* A.H. Sm.), sect. *Fulviglebae* (*R. diabolicus* A.H. Sm., *R. ochraceisporus* A.H. Sm., *R. vinicolor* A.H. Sm.), and sect. *Villosuli* (*R. colossus* A.H. Sm., *R. fragrans* A.H. Sm., *R. hawkerae* A.H. Sm.).

Table 1 sets forth the classification of the taxa listed in Appendix 1 according to Smith & Zeller (1966) and Grubisha et al. (2002). Many taxa are not included in Table 1, since neither Smith & Zeller (1966) nor Grubisha et al. (2002) studied the specimens.

Morphological characters

New collections were identified according to morphological criteria following Martín (1996). Spore volumes were calculated (Groβ & Schmitt 1974) for collections representing the *Rhizopogon roseolus* group (Table 2), with volumes calculated for each specimen in collections with more than one basidiome (e.g. 3ROS to11ROS).

Molecular taxonomic methods

Genomic DNA was extracted using an E.Z.N.A. Fungi DNA miniprep kit (Omega Biotek, Doraville, USA) as described in Martín & García-Figueres (1999). DNA fragments containing internal transcribed spacers ITS1 and ITS2 were amplified with direct universal primers ITS1F or ITS1 and the reverse primer ITS4 as described in Martín & Raidl (2002). Prior to sequencing, the amplification products were cleaned using QIAquick gel PCR purification kit (QIAGEN, Valencia, California, USA). When more than 20 ng/μl were obtained, both strands were sequenced separately using primers mentioned above at Secugen S.L. (Madrid, Spain). However, when weak products were visualized on agarose gels, the products were cloned using pGEM®-T Easy Vector System II cloning kit (Promega Coorporation, Madison. Wisconsin, USA). From each cloning reaction 3 clones were selected for sequencing. To confirm that the

Table 1. Comparison of Smith & Zeller (1966) and Grubisha et al. (2002) classification of taxa included in this study.

Smith & Zeller (1966)			Taxa	Grubisha et al. (2002)*
Sect. *Amylopogon*			*R. atroviolaceus*	n.d.
			R. ellenae	Subgen. *Amylopogon*
Sect. *Fulviglebae*			*R. diabolicus* *R. ochraceisporus* *R. vinicolor*	Subgen. *Villosuli* sect. *Vinicolores*
Sect. *Villosuli*			*R. colossus* *R. hawkerae*	Subgen. *Villosuli* sect. *Villosuli*
			R. fragrans	n.d.
Sect.	Subsect.	Stirps		
Rhizopogon	*Angustipori*	*Ochraceorubens*	*R. ochraceorubens*	Subgen. *Rhizopogon*
		Vulgaris	*R. vulgaris*	Subgen. *Roseoli*
	Rhizopogon	*Luteolus*	*R. luteolus*	Subgen. *Rhizopogon*
		Rubescens	*R. abietis* *R. luteorubescens* *R. ochroleucoides* *R. pseudoroseolus*	n.d.
			R. roseolus	Subgen. *Roseoli*
			R. rubescens var. *rubescens* *R. rubescens* var. *ochraceus* *R. rubescens* var. *pallidimaculatus* *R. ventricisporus*	n.d.

*Taxa not included in Grubisha et al. (2002) are indicated as n.d. (no data).

inserted product was correct, prior to sequencing, 2 µl of the purified plasmid DNA was digested with EcoRI according to manufacturer instructions. Both strands were sequenced separately using vector specific primers T7 and SP6 at Secugen S.L.
Sequencher (Gene Codes Corporations, Ann Arbor, Michigan, USA) was used to identify the consensus sequence from the two strands of each ITS nrDNA isolate. Blastn searches with megablast option were used to compare the sequences obtained against the sequences in the National Center of Biotechnology Information (NCBI) nucleotide databases. New consensus sequences have been lodged in the EMLB-EBI database with the accession numbers indicated in Appendix 1.

SEQAPP software for multiple sequences was used to search for the best alignment. Two alignments were created. Alignment 1 included sequences obtained from taxa cited in Appendix 1. Alignment 2, generated after analyzing the first alignment, included only

sequences grouped in the *R. roseolus* complex clade and selected sister group sequences. Where alignment ambiguities, the alignment chosen was the one generating the fewest potentially informative characters. Alignment gaps were marked as "–", unresolved nucleotides and unknown sequences were indicated with "N". Alignment were analysed using PAUP*Version 4.0b10 for Macintosh (Swofford 2003) and MrBAYES 3.0 7 (Huelsenbeck & Ronquist 2001).

Maximum parsimony analyses (MP) were inferred using the heuristic search option in PAUP*4.0b10. Gaps were treated as missing data. Branch lengths equal to zero were collapsed to polytomies. Nonparametric bootstrap (bs) support (Felsenstein 1985) for each clade was tested based on 10.000 replicates, using the fast-step option. The consistency index CI (Kluge & Farris 1969), retention index RI (Farris 1989), and rescaled consistency index RC (Farris 1989) were obtained. Four alignment analyses were performed (Table 3): 1) alignment of all obtained sequences (both complete and incomplete) and all characters, including ambiguities; 2) alignment of all obtained sequences (both complete and incomplete) but excluding the part of the alignment with ambiguities; 3) alignment of only complete sequences and excluding ambiguities, 4) alignment of only complete sequences but without excluding ambiguities. In Alignment 2, incomplete sequences were eliminated and no ambiguities were obtained, so only one analysis was done.

Bayesian analyses of the two alignments (all taxa and all characters) were done separately using MrBAYES 3.0 (Huelsenbeck & Ronquist 2001) following Huelsenbeck et al. (2000) and Larget & Simon (1999). Posterior probabilities (pp) were approximated by sampling trees using a Markov Chain Monte Carlo (MCMC) method. Posterior probabilities of each branch were calculated by counting the frequency of trees that were visited during the course of the MCMC analysis. The analysis was performed assuming the general time reversible model (Rodríguez et al. 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six categories (GTR+I+G). No molecular clock was assumed. A run with 2,000,000 generations starting with a random tree and employing 12 simultaneous chains was executed. Every 100th tree was saved into a file for a total of 20,000 trees. We plotted the log-likelihood scores of sample points against generation time using TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.html?I=tracer) and determined that stationarity was achieved when the log-likelihood values of the sample point reached a stable equilibrium value (Huelsenbeck & Ronquist 2001). The initial 1,000 trees were discarded as burn-in before stationarity was reached. Using the MrBAYES "sumt" command, majority-rule consensus trees were calculated from 19,000 trees sampled after reaching likelihood convergence to calculate the posterior probabilities of the tree nodes. Phylogenetic trees were drawn using TreeView (Page 1996).

Only supported (bs>0.85 and/or pp> 0.95) monophyletic terminal clades were considered for determining a phylogenetic species.

Results

From some samples (*R. abietis, R. inodorus, R. lapponicus, R. minor, R. ochroleucoides, R. pumilionus, R. roseolus* f. *aberrans, R. rubescens* var. *ochraceus, "R. tenuisporus* var. *intermedius", R. ventricisporus*) it was not

Table 2. Possible host and spore volume of taxa under the *Rhizopogon roseolus* complex clade (Figs. 1–2).

Taxon[1]	Code	Possible host[1]	Spore Volume[2]
R. gigasporus	1GIG	*P. pinaster*	E
R. graveolens f. *pomaceus*	1POM	*Pinus* spp.	B
R. luteorubescens	1LRU	*P. contorta*	C
R. minor	1MIN	*Carpinus* sp.	B
R. mohelnensis	1MOH	*Abies* sp., *P. sylvestris*	D
R. pseudoroseolus	1PSE	*P. resinosa*	C
	2PSE	*P. resinosa*	C
	3PSE	*P. resinosa*	C
R. roseolus	1ROS	*Pinus* spp.	n.d.
	2ROS	*P. contorta*	B
	3ROS–11ROS	*Pi. abies*	C,–,D, B,C,B,B,C, B
	12ROS	*Abies alba*	D
	13ROS	*Ca. sativa*, *P. pinaster*, *Q. pyrenaica*	B
	14ROS	*P. sylvestris*	D
	15ROS	under *Cistus* sp.	C
	16ROS	*P. nigra* /*F. sylvatica*	D
	18ROS	*P. sylvestris*	D
	19ROS-20ROS	*P. sylvestris*	C, C
	21ROS-22ROS	*P. sylvestris*	D, B
	23ROS-28ROS	*P. sylvestris*	C, B, C, B, C, B
	29ROS	*P. sylvestris*	D
	30ROS-31ROS	*P. sylvestris*, *P. nigra*	B, B
	32ROS-35ROS	*P. sylvestris*, *P. nigra*	A, B, A, B
	36ROS	Pine forest under *Q. ilex*	C
	37ROS	*P. sylvestris*	D
	38ROS	*P. pinaster*	C
	39ROS	*P. pinaster*	B
	40ROS	*P. pinaster*,*Q. ilex*	B
	41ROS	*P. sylvestris*, *Picea* sp., *Larix* sp.	D
	42ROS	*Pinus* sp.	n.d
	43ROS	*P. sylvestris*	n.d
	44ROS	*P. sylvestris*, *Q. robur*, *Co. avellana*	n.d
R. rubescens	1RUB	*P. muricata*	n.d
	3RUB	*P. sylvestris*	C
	4RUB	*P. sylvestris*	n.d.
	5RUB	*P. sylvestris*	n.d.
R. rubescens var. *pallidimaculatus*	1PAL	*Abies* sp., *Pinus* sp.	B
R. sardous	1SAR	Mixed forest: *Pinus* sp., *Eucalyptus* sp.	B
R. vulgaris	1VUL	*Pinus* sp.	n.d.
	2VUL	*Pinus* sp.	n.d.

1. Abbreviations: *Ca.* = *Castanea*; *Co.* = *Corylus*; *F.* = *Fagus*; *Pi.* = *Picea*; *P.* = *Pinus; Q.* = *Quercus.*
2. In sample 4ROS almost all spores lacking an exosporium; n.d. = size data lacking.

possible to get good sequences, even after cloning. In others (*R. duriusculus, R. graveolens* f. *pomaceus, R. roseolus* var. *foetens* and *R. tenuisporus*), the sequences obtained were so different from the rest of *Rhizopogon* that was not possible to include them in the alignment; the Blast search gave a very high score to sequences of *Boletus* sp. and/or *Coniophora puteana* (Schumach.) P. Karst.; in APPENDIX 1, this is indicated with a question mark.

The 50 new ITS sequences obtained in this study are listed (with GenBank accession numbers) in Appendix 1. Eleven sequences were derived from type material. The sequences were aligned and analyzed together with 28 other taxa representing sect. *Rhizopogon* subsects. *Rhizopogon* (stirps *Rubescens* & *Luteolus*) and *Angustispori* (stirps *Vulgaris* & *Ochraceorubens*), sect. *Villosuli*, sect. *Fulviglebae,* and sect. *Amylopogon* obtained in previous studies (Johanesson & Martín 1999, Martín & Raidl 2002) or by other authors (Taylor & Bruns 1999, Grubisha et al. 2002, Kretzer et al. 2003, Menkis et al. 2005, Tedersoo et al. 2006, Fransson et al. 2007).

Alignment 1

The complete ITS dataset included 69 sequences and 840 characters (Alignment 1). The values of the 4 parsimony analyses are summarized in TABLE 3. In the B/MCMC analysis of the combined data set, the likelihood parameters in the sample had the following mean (Variance): LnL = –3449,377 (0.053), base frequencies π(A)= 0.254 (0.0003), π(C) = 0.205 (0.0004), π(G) = 0.232 (0.0003), π(T) = 0.309 (0.0003), rate matrix r(AC) = 2.048 (0.003), r(AG) = 4.551 (0.003), r(AT) = 2.72 (0.003), r(CG) = 1.391 (0.004), r(CT) = 7.104 (0.002), r(GT) = 1, the gamma shape parameter alpha alpha = 0.109 (0.002), and the proportion of invariable site p(invar) =0.338 (0.0003).

The topologies of the four MP analyses were similar to each other (not shown), and also to the B/MCMC tree (FIG. 1).

Rhizopogon luteolus (Smith & Zeller 1966: sect. *Rhizopogon,* stirps *Luteolus*) and *R. ochraceorubens* (Smith & Zeller 1966: Sect. *Rhizopogon,* stirps *Ochraceorubens*), both from subgen. *Rhizopogon*, were basal to the other *Rhizopogon* taxa studied.

One *R. rubescens* collection (2RUB, distributed as Ellis North Amer. 943 Exsiccati and described as *R. rubescens* sensu A.H. Sm.) was basal to the well-supported clade (99% bs/1.00 pp) that comprised collections of subgen. *Amylopogon* and subgen. *Villosuli* as well as the remaining taxa studied.

The *R. roseolus* complex clade (subgen. *Roseoli* in Grubisha et al. 2002) was sister (75% bs/1.0 pp) to the highly supported (93% bs/1.0 pp) clade formed by 6 taxa from subgen. *Villosuli* (3 from sect. *Villosuli* and 3 from *Fulviglebae* in Smith & Zeller, 1966). However, the sister-group relationship was not well supported (63% bp /0.68 pp).

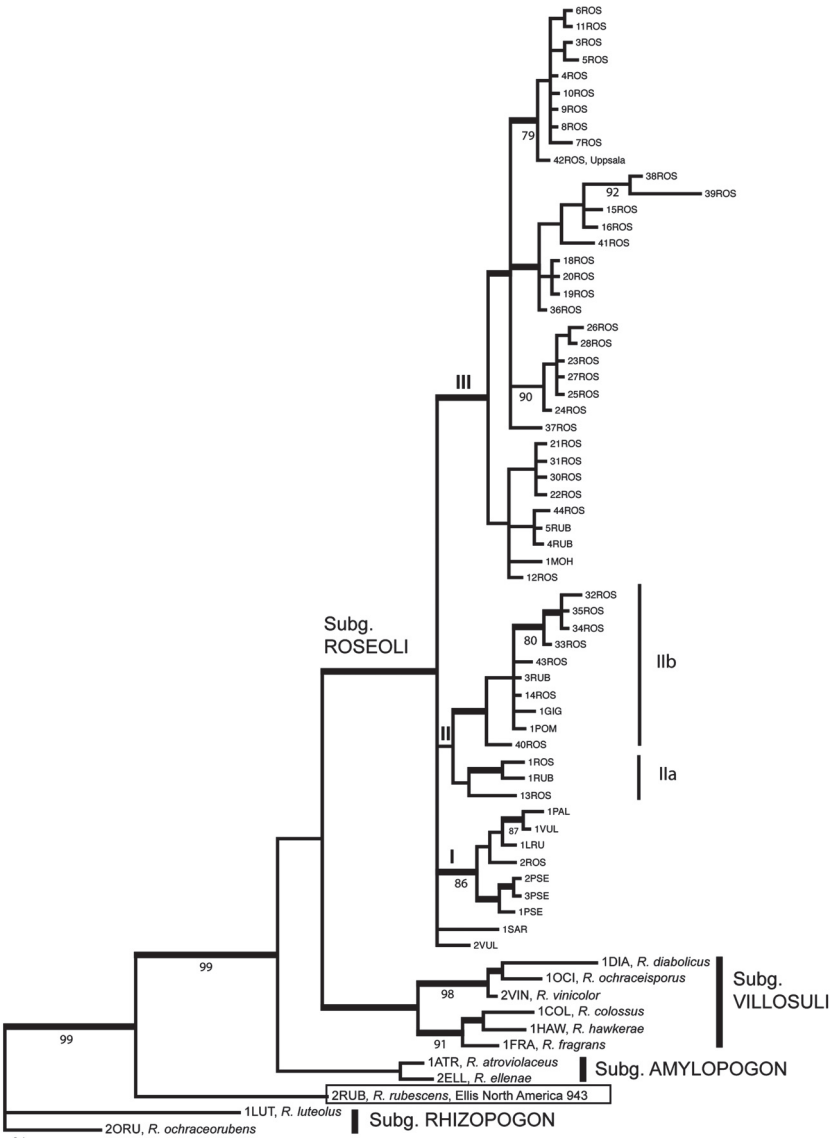

Fig. 1. Majority rule consensus tree from Bayesian analysis of *Rhizopogon* taxa cited in Appendix 1 (Alignment 1). Branches with posterior probabilities superior to 90 are indicated in bold. Bootstrap values superior to 50% are indicated under the branches. The analysis separated collections of *Rhizopogon* subgen. *Roseoli* into at least five clades.

Table 3. Maximum parsimony analyses of the *Rhizopogon roseolus* group.

	analysis 1[a]	analysis 2[b]	analysis 3[c]	analysis 4[d]
Total characters	840	740	740	840
Constant characters	624	542	547	629
Parsimony-uninformative characters	98	84	85	99
Parsimony-informative characters	118	114	108	112
Tree length	347	325	306	328
Consistency index (CI)	0.7579	0.7477	0.7647	0.7744
Homoplasy index (HI)	0.2421	0.2523	0.2353	0.2256
Rescaled Index (RI)	0.8791	0.8794	0.8837	0.8833
Rescaled consistency index (RC)	0.6663	0.657	0.6758	0.6840

[a] all sequences and characters included
[b] all sequences included; ambiguous characters excluded
[c] only complete sequences included; ambiguous characters excluded
[d] only complete sequences included; ambiguous characters included

The *R. roseolus* complex collections fell into three main clades (I, II, III). Clade II was not well supported, however, and the relationship of one *R. sardous* collection and one *R. vulgaris* collection (2VUL from US) to the other *R. roseolus* complex collections included in the clade was not resolved.

Clade I contained seven sequences obtained from US samples collected under *Pinus* spp. (Table 2): *R. roseolus* sensu AH. Sm. (2ROS), *R. pseudoroseolus* (1PSE-3PSE), *R. luteorubescens* (1LRU) and one sequence of *R. vulgaris* (1VUL). The sequence produced from a sample of *R. rubescens* var. *pallidimaculatus* (1PAL) collected under *Abies* sp. clustered in this clade. Only two spore volume ranges were obtained (B and C).

Clade II was not well supported (0.61 pp) and was composed of two subgroups. Clade IIa included two sequences from California (US), identified as *R. roseolus* (1ROS) and *R. rubescens* (1RUB), and a sample from Spain (13ROS) collected under *Castanea sativa* Mill. in a mixed forest of *Pinus pinaster* Aiton and *Quercus pyrenaica* Willd. Clade IIb was represented by samples (14ROS, 32ROS-34ROS, 40ROS) collected under *Pinus sylvestris* L. in Spain, a possible *R. rubescens* (3RUB) collected close to a *R. roseolus* (14ROS) and two type collection sequences (*R. graveolens* f. *pomaceus,* 1POM; *R. gigasporus*, 1GIG, newly described from Italy in 1984 and with abnormally large spores).

Clade III contained European *R. roseolus* samples from Slovenia (3ROS-9ROS) collected under *Picea abies* (L.) H. Karst., samples from Italy under *Pinus pinaster* (38ROS-39ROS) and *P. nigra* J.F. Arnold (16ROS), many Spanish samples collected under *P. sylvestris,* and two samples collected under *Abies*

alba Mill. (one obtained from the *R. mohelnensis* type collection). Moreover, two sequences obtained from ectomycorrhizal root tips and identified as *R. rubescens* (4RUB and 5RUB) clustered in this group. Spore volumes ranged from ~30 μm³ (A, 12 collections) through ~ 45μm³ (B, 10 collections), and ≤ 60 μm³ (C, 8 collections). No other spore volume data were obtained, although collapsed spores lacking an exosporium were found in collection 4ROS.

Alignment 2

In order to improve the resolution and refine alignment in the *R. roseolus* complex clade (Subg. *Rhizopogon*), the following sequences were excluded — *R. ochraceorubens* (2ORU), *R. luteolus* (1LUT), Ellis North Amer. 943 Exsiccati (2RUB), *R. ellenae* (2ELL), *R. atroviolaceus* (1ATR), and *R. roseolus* clade collections with incomplete sequences. Here six subgen. *Villosuli* sequences were included as outgroup. The Alignment 2 complete ITS dataset included 53 sequences and 679 non-ambiguous characters among which 546 characters were constant and 92 parsimony informative. Maximum parsimony (MP) analysis under heuristic search gave 100 most parsimonious trees with a length of 214steps, CI= 0.7430, RI= 0.9113 and RC= 0.6771.

In the B/MCMC analysis the likelihood parameters in the sample had the following mean (Variance): LnL = –2464,531 (0.009), base frequencies π(A)= 0.243 (0.0004), π(C) = 0.22 (0.0004), π(G) = 0.238 (0.0004), π(T) = 0.3 (0.0004), rate matrix r(AC) = 0.929 (0.003), r(AG) = 4.857 (0.003), r(AT) = 1.79 (0.004), r(CG) = 1.796 (0.003), r(CT) = 5.576 (0.003), r(GT) = 1 , the gamma shape parameter alpha = 0.109 (0.002), and the proportion of invariable site p(invar) =0.539 (0.0003).

The MP and B/MCMC tree topologies were similar; only the Bayesian tree is shown in Fig 2. The specimens under *R. roseolus* complex clade (subgen. *Roseoli*) clustered in a very well supported clade (100% bs/ 1.0 pp). Alignment 1 and 2 phylogenetic analyses place sequences in almost the same main groups but show different relationships within the clades.

Again, the *R. sardous* specimen was not related to the other taxa in the *R. roseolus* complex group; however, the *R. vulgaris* collection (2VUL from US) grouped into clade I (all taxa from North America). Clade I related with two well-supported clades (IIa and III), although the relationship between them was ambiguous (< 50%/ 0.56 pp). The group formed by these clades was sister to clade IIb and the *R. sardous* specimen.

These ITS sequence analyses imply at least five possible phylogenetic "species" — *R. sardous*, clade I, clade IIa, clade IIb, and clade III. Moreover, two groups appear to have a North American affiliation (clade I and clade IIa, except isolate 13ROS from Spain), both closely related to *R. roseolus* sensu stricto (clade III).

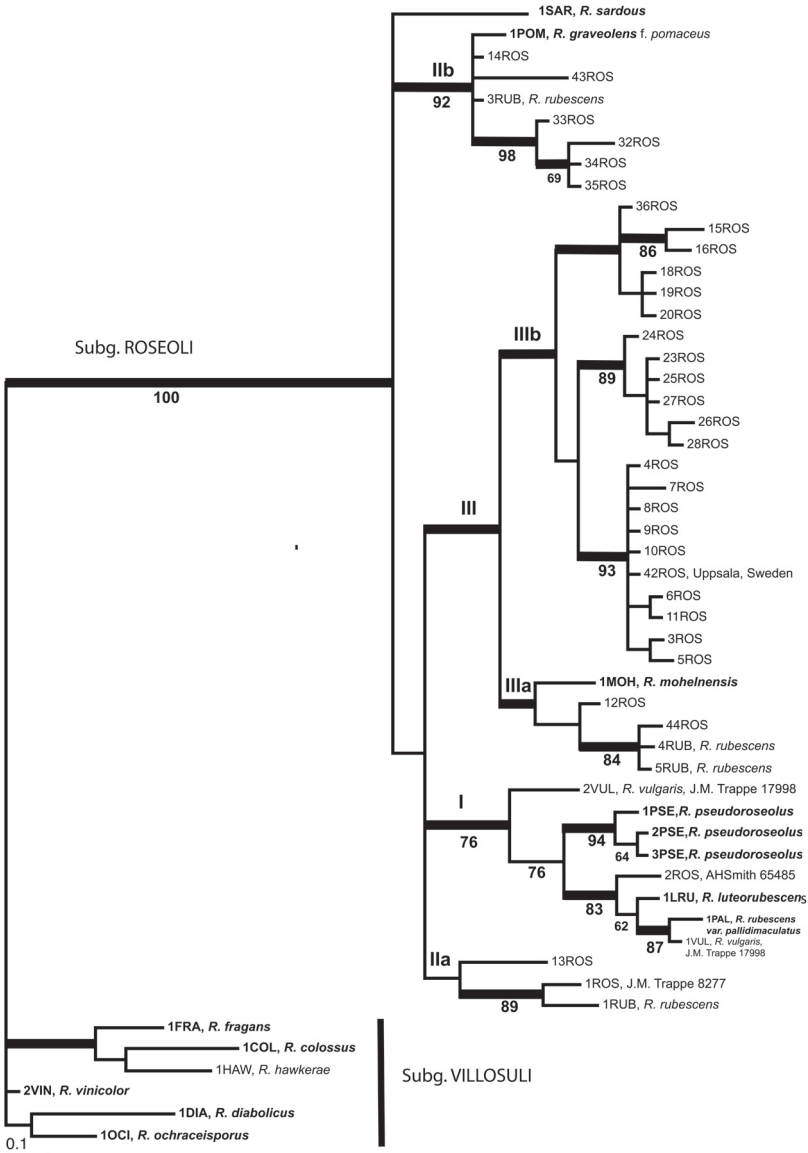

Fig. 2. Majority rule consensus tree from Bayesian analysis of *Rhizopogon* taxa cited in Appendix 1 (Alignment 2). Branches with posterior probabilities superior to 90 are indicated in bold. Bootstrap values superior to 50% are indicated under the branches. The analysis separated collections of *Rhizopogon* subgen. *Roseoli* into at least five clades.

Discussion

Molecular variability of *R. roseolus* sensu M.P. Martín

The above analyses separated collections of *R. roseolus* complex into at least five clades indicative of five possible phylogenetic species. These findings conflict taxonomically with the ~37 names proposed for this group. We note, however, that molecular data have been generated for only 11 of those 37 taxa.

Rhizopogon sardous was collected in West Sardinia beneath *Eucalyptus* and *Pinus* in sandy soil. According to Pacioni (1984b), the peridium is simple, but the external hyphae (the outer 20–80 μm) are pigmented and become pink to purple in Cresyl Blue, whereas the inner (100–120 μm) hyphae show extracellular pigments and become blue-green in Cresyl Blue. Since the Cresyl Blue reaction cannot be tested in dry specimens and no other morphological differences were observed, Martín (1996) considered *R. sardous* synonymous to *R. roseolus*. Although more fresh collections should be studied and host (*Eucalyptus* or *Pinus*) specificity level should be tested, our molecular data strongly support *R. sardous* as a distinctive species. As mentioned in the Kjøller & Bruns (2003) study on *Rhizopogon* species in spore bank distributions across five California pine forests, isolation may drive *Rhizopogon* diversification.

Clade I. Samples included in clade I have a North American affiliation. Three taxa — *R. luteorubescens*, *R. pseudoroseolus* and *R. rubescens* var. *pallidimaculatus* — described in Smith & Zeller (1966) cluster in this clade with a "*R. roseolus* sensu A.H. Sm." collection. Two other collections (identified by J.M. Trappe as *R. vulgaris*) grouped with these collections; the sequence from one specimen (1VUL) is close to *R. rubescens* var. *pallidimaculatus* except for 2 base shifts (1PAL/1VUL) [alignment 1: position 7 (C/Y) and position 281 (G/A)]. A check of the "*R. vulgaris*" descriptions reveal no clear diagnostic characters except for some peridium and gleba colour variability including colour changes in those pseudotissues resulting from application of $FeSO_4$ and KOH. In *R. pseudoroseolus* the white gleba suggests immature basidiomes; the basidiomes described as *R. luteorubescens* appear immature, with a white, pallid yellowish to ochraceous gleba. However, the *R. rubescens* var. *pallidimaculatus* gleba colour description seems to refer to more mature basidiomes. Smith & Zeller (1966) included these taxa in subg. *Rhizopogon* sect. *Rhizopogon* subsect. *Rhizopogon* stirps *Rubescens*, where also they described *R. roseolus* sensu A.H. Sm., a taxon that does not group with any *R. roseolus* European collection. The low ITS variation within these taxa may reflect lack of resolution in this region (Bidartondo & Bruns, 2002), but our review of the macroscopical and microscopical characters implies that all these taxa could represent one distinctive North American species. Likewise, the two collections of *R. vulgaris* sensu Trappe seem to belong to this phylogenetic species. Determination of the correct name is premature,

however, until the other taxa mentioned in Smith & Zeller (1966) undergo phylogenetic analyses. Studies comparing ectomycorrhizae are also needed to clarify this group, since we have very few data related to the specific hosts. Our work, as well as that by authors before us (Martín et al. 1998, Bidartondo & Bruns 2002, Kjøller & Bruns 2003), confirms that the application of names to *Rhizopogon* collections has been very inconsistent.

Clade IIa. This clade, which comprises only two sequences from North America and one sample from Spain (13ROS), needs additional data to improve resolution; however, we feel the clade may represent a distinctive phylogenetic species. The 1RUB sequence is derived from an unidentified ectomycorrhizal basidiome with putative suilloid morphology (isolate RCP-13 in Taylor & Bruns 1999) that molecular data place close to *R. rubescens*. Molecular analysis by Grubisha et al. (2002) supports the 1ROS sequence, obtained from a Trappe collection identified as *R. roseolus*, in a clade (87% bs) closely related to *R. burlinghamii* (subgen. *Rhizopogon* sect. *Rhizopogon* subsect. *Angustipori* stirps *Ochraceorubens*). The *R. burlinghamii* sequence located at the GenBank (AF058303) belongs to a collection from J.M. Trappe (JMT17882), not to the type collection (Zeller Herb. 8244). It is necessary to obtain the ITS sequence of the *R. burlinghamii* type collection and to compare it with the sequences in clade IIa to ascertain whether or not collections in this clade belong to the species, *R. burlinghamii*.

Clade IIb. This clade represents European specimens associated primarily with *Pinus sylvestris* and *P. nigra,* exceptions being *R. gigasporus* (*P. pinaster*) and *R. graveolens* f. *pomaceus* (*Pinus* spp.). According to Vidal (1991), specimens of *R. roseolus* sensu stricto are associated primarily with *Pinus halepensis* Mill. and *P. sylvestris* in calcareous soils, whereas specimens of *R. rubescens* sensu stricto are found near *Pinus pinea* L. and *P. pinaster* in sandy soil. In Europe is difficult to find, at least in the Mediterranean, forests containing only one pine species. Our recent morphological revision shows that specimens in this clade have thin peridia and a high concentration of extracellular red pigments, which support these collections as belonging to *R. rubescens* sensu stricto. Thus *R. gigasporus* and *R. graveolens* f. *pomaceus* are confirmed as synonyms of *R. rubescens*.

Clade III. Sequences obtained from specimens from Slovenia, Lithuania, Portugal, Spain, and Sweden are included in this clade. Based on our sampling, clade III could correspond to *R. roseolus* sensu stricto. Corda's original description of *Splanchnomyces roseolus* was based on specimens collected from Praha (Czech Republic) under *Pinus sylvestris*. In this clade, only the sequence of *R. mohelnensis* (1MOH), collected in Czech Republic under *Pinus sylvestris* was included, which should be regarded as a synonym of *R. roseolus* sensu stricto. When immature, in *R. roseolus* the peridium is pure white, then yellow

to pink; rubbing turns the peridium red to deep purple. Although many of the collections fruited under *Pinus sylvestris,* other possible hosts (*P. nigra*, *P. pinaster* and *Picea abies*) are mentioned in the herbarium labels.

We found that in Menkis et al. (2005) the sequences were not well identified, since the strain NA202A (43ROS) belongs to the *R. rubescens* clade and strains "aurim738" (4RUB) and "aurim NS182" (5RUB) belong to *R. roseolus*.

Relationships in Smith & Zeller sect. *Rhizopogon*

Even though, the collections mentioned in Smith & Zeller (1966) are immensely valuable to *Rhizopogon* taxonomic and phylogenetic studies, our results reconfirm that the different subsections and stirps in sect. *Rhizopogon,* as based on peridium and gleba colours and basidiospore sizes, do not form monophyletic clades. Grubisha et al. (2002) taxonomic revision of the subgenera was a very important contribution.

Reexamination of all material distributed in the Ellis North America 943 Exsiccati is needed to reclassify the species. Zeller & Dodge (1918) included this collection under both *R. roseolus* (exsiccati in Mo. Bot. Gard. Herb and Burt Herb.) and *R. rubescens* (exsiccati in U.S. Dept. agr., Bur.PL, Ind. Path. Coll). Smith & Zeller (1966) also assigned the same exsiccati number (but located at NYBG and source for our own sequence) to *R. rubescens*.

Conclusions

As mentioned by Bidartondo & Bruns (2002) with respect to the genus *Gautieria*, many conflicts in the *R. roseolus* complex phylogeny probably stem from taxonomic concepts that vary from one continent to another. Collaborations among scientists around the world are required to solve the conflicts in species limits in *Rhizopogon*, as well as in other fungi.

Many papers published during recent years apply molecular taxonomic methods to study the ectomycorrhizal (EM) community structure, such as identifying resistant *Rhizopogon* propagules in post fire communities. However, before making conclusions, it is very important to ensure that sequences located at the public databases (such as EMBL, UNITE) are obtained from well-identified specimens. The taxonomic studies, although not always well funded and frequently neglected, are the basic to many applied research and application.

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Appendix 1. *Rhizopogon* DNA sequences analyzed in this study.

Commas separate 8 elements in the appendix below: **1**—taxon name (in accordance with *Index Fungorum*) taken from the original collection label or reference (journal, book, GenBank); an asterisk (*) indicates a name not included in Smith & Zeller (1966) or Grubisha et al. (2002) and regarded as synonymous with *R. roseolus* in Martín (1996). **2**— *Rhizopogon* spp. database alphanumeric codes (see also Figs. 1–2). **3**— collection number. **4**— herbarium acronym (see Holmgren et al. 1990). **5** (when present)—type status. **6**— collection area. **7**—country code (ISO 3166 via acronymsearch.com). **8**— GenBank accession numbers, with sequences obtained during this study in **bold** and those taken from GenBank referenced as follows: [a] = Grubisha et al. 2002, [b] = Johanesson & Martín 1999, [c] = Kretzer et al. 2003, [d] = Martín & Raidl 2002, [e] = Fransson et al. 2007, [f] = Menkis et al. 2005, [g] = Tedersoo et al. 2006, [h] = Taylor & Bruns 1999; * denotes nrDNA ITS 1 (complete) + 5.8 S (partial) and ** denotes nrDNA 5.8S (partial) + ITS2 (complete). **?** = *Boletus lupinus* Fr. and/or *Coniophora puteana* given as best BLAST scores. – = no sequence obtained.

R. atroviolaceus, 1ATR, AHS 69179, E , paratype, Idaho, US, **AM085520***.
R. abietis, 1ABI, AHS 69834, MICH, paratype, Idaho, US, –.
R. abietis, 2ABI, MPM 530, BCC, Girona, ES, –.
R. colossus, 1COL, AHS 49480, MICH, holotype, Oregon, US, AF071441[a], AF071442[a].
R. diabolicus, 1DIA, AHS 68424, MICH, paratype, Idaho, US, AF071444[a], AF071443[a].
*R. duriusculus**, 1DUR, PRM 154791, holotype, Mnichovice, CZ, **?** .
R. ellenae, 2ELL, AHS 66137, MICH, holotype, Idaho, US, AF071445[a], AF071446[a].
R. fragrans, 1FRA, AHS 60155, MICH, paratype, Idaho, US, **AM085523**.
*R. gigasporus**, 1GIG, AQUI, holotype, Tabarka, TN, **AJ810044***.
R. graveolens f. *pomaceus**, 1POM, PRM 619028, isotype, Právcice, CZ, **AJ810037**.
R. graveolens f. *pomaceus**, 2POM, PRM 619033, isotype, Právcice, CZ, **?**.
R. hawkerae, 1HAW, AHS 68417, MICH, Washington, US, AF071447[a], AF071448[a].
*R. inodorus**, 1INO, PRM 148574, Jilové, CZ, –.
*R. lapponicus**, 1LAP, P.A. Karsten 3695, H, type, Rossia Karelia, FI, –.
*R. lapponicus**, 2LAP, P.A. Karsten 3696, H, type, Rossia Karelia, FI, –.
R. luteolus, 1LUT, JMT 22516, OSC, Uppsala, SE, AF062936[a].
R. luteorubescens, 1LRU, AHS 58778, MICH, holotype,Idaho, US, **AJ810038**.
R. minor, 1MIN, PRM 154798, type, Mnichovice, CZ, –.
R. mohelnensis, 1MOH, PRM 154616, type, Moheno, CZ, **AJ810039, ?**.
R. ochraceisporus,1OCI,AHS65963, MICH, paratype,Idaho, US,AF071439[a].
R. ochraceorubens, 2ORU, AHS 59643, MICH, holotype, Idaho, US,AF062928[a].
R. ochroleucoides, 1OLE, AHS 68310, MICH, paratype, Idaho, US,– .
R. pseudoroseolus, 1PSE, AHS 66302, MICH, paratype, Michigan, US, **AJ810040**.
R. pseudoroseolus, 2PSE, AHS 66604, MICH, paratype, Michigan, US, **AJ810041**.
R. pseudoroseolus, 3PSE, AHS 66469a, MICH, paratype, Michigan, US, **AJ810042**.
R. pumilionus *, 1 PUM–2PUM, ex herb Soehner 1184, M, type, DE, –,–.
R. roseolus sensu Trappe, 1ROS, JMT 8227, OSC, California, US, AF058315[a].
R. roseolus sensu A.H. Sm., 2ROS, AHS 65485, MICH, Idaho, US, **AJ810045**.
R. roseolus sensu M.P. Martín, 3ROS–11ROS, 1–9 MPM2714, MA–Fungi 47710, Gozd Martuljek, SI, **AJ810046–AJ810054**.
*R. roseolus,*12ROS, MPM2717, MA-Fungi 47711, Huesca, ES, **AJ810055**.
*R. roseolus,*13ROS, MPM2725, MA-Fungi 47688, Ávila, ES, AJ419209[d].

*R. roseolus,*14ROS, MPM2819, MA-Fungi 47689, Girona, ES, AJ419211[d].

*R. roseolus,*15ROS, MPM2858, MA-Fungi 47687, Estremadura, PT, AJ419210[d].

*R. roseolus,*16ROS–17ROS, MPM2898, MA-Fungi 47712, Tarragona, ES, **AJ810056, –**.

*R. roseolus,*18ROS, MPM2911, MA-Fungi 47713, Castellón, ES, **AJ810057**.

*R. roseolus,*19ROS–20ROS,1–2 MPM2912, MA-Fungi 47714, Castellón, ES, **AJ810058, AJ810059**.

R. roseolus, 21ROS–22ROS, 1–2 MPM2913, MA-Fungi 47715, Castellón, ES, **AJ810060**, AJ810061****.

R. roseolus, 23ROS–28ROS, 1–6 MPM2917, MA-Fungi 47716, Tarragona, ES, from **AJ810062** to **AJ810067**.

R. roseolus, 29ROS, MPM2918, MA-Fungi 47717, Tarragona, ES, **–**.

R. roseolus, 30ROS–31ROS, 1–2 MPM2921, MA-Fungi 47718, Tarragona, ES, **AJ810068**, AJ810069****.

R. roseolus, 32ROS–35ROS, 1–4 MPM2922, MA-Fungi 47719,T arragona, ES, from **AJ810070** to **AJ810073**.

R. roseolus, 36ROS, MPM2928, MA-Fungi 47720, Castellón, ES, **AJ810074**.

R. roseolus, 37ROS, MPM 1511, BCC, Mallorca, ES, AF115840**[b].

R. roseolus, 38ROS, Sarasini 286, S. Vicenzo, IT, AF115841**[b].

R. roseolus, 39ROS, Sarasini 451, Marina di Vecchiano, IT, AF115842**[b].

R. roseolus, 40ROS, Sarasini 521, Girona, ES, AF115843**[b].

R. roseolus, 41ROS, Sarasini 612, Fondo, IT, AF115844**[b].

R. roseolus, 42ROS, isolate RrUP175 (ectomycorrhiza root tip), Uppsala, SE, DQ179127[e].

R. roseolus, 43ROS, strain NA202A (ectomycorrhiza root tip), LT, DQ068964[f].

R. roseolus, 44ROS, L999, TAA 185325 , Karuse, EE, AJ966744[g].

R. roseolus f. *amygdaloporus**, 1AMY, UPS, Närke, SE, **–**.

R. roseolus f. *aberrans**, 1ABE, UPS, Uppland, SE, **–**.

R. roseolus f. *foetens**, 1FOE, PRM 618989, Pradice, CZ, **?**.

R. rubescens, 1RUB, RPC-13 (ectomycorrhiza root tip),California, US, AF158018[h].

R. rubescens, 2RUB, Ellis North Amer. 943 Exsiccati, M, New Jersey, US, **AJ810034**.

R. rubescens, 3RUB, MPM2815, MA-Fungi 47730, Girona, ES, **AM085528**.

R. rubescens, 4RUB, isolate aurim738 (ectomycorrhiza root tip), LT, DQ069016[f].

R. rubescens, 5RUB, isolate NS182 (ectomycorrhiza root tip), LT, DQ068965[f].

R. rubescens var. *ochraceus*, 1OCH, AHS 60079, UPS, paratype, Idaho, US, **–**.

R. rubescens var. *ochraceus,* 2OCH, AHS 59481, UPS, paratype, Idaho, US, **–**.

R. rubescens var. *pallidimaculatus,* 1PAL, AHS 58821a, MICH, holotype, Idaho, US, **AJ810043**.

*R. sardous**, 1SAR, AQUI, Sardegna, IT, **AM085529**.

*R. tenuisporus**, 1TEN, PRM 19570, lectotype, Sobeslav-Blata, CZ, **?**.

*R. tenuisporus**, 2TEN, PRM 154619, sintype, Sobeslav-Blata, CZ, **?**.

"R. tenuisporus var. *intermedius"**, 3TEN, PRM 742575, type, Bzenec, CZ, **?**,**–**.

R. ventricisporus, 1VEN, AHS 69165, MICH, holotype, Idaho, US, **–**.

R. vinicolor, 2VIN, Trueblood 2195, MICH, holotype, Idaho, US, **AJ810035**.

R. vulgaris, 1VUL, JMT 19154, OSC, Oregon, US, AF062934[a].

R. vulgaris, 2VUL, JMT 17998, OSC, California, US, AF062931[a].